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# Application of 5-HT-SO<sub>4</sub> in Biomarker Research

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## Abstract

A serotonin catabolite, serotonin O-sulphate (5-HT-SO<sub>4</sub>), is hypothesised to accentuate the intensity of serotonin metabolism in the central nervous system (CNS). We hypothesised that serotonin O-sulphate could be quantified in human plasma using modern liquid chromatography-mass spectrometry. To test our hypothesis, we performed a critical literature review and a three-stage trial. First, a suitable liquid chromatography-mass spectrometry (LC-MS/MS) method for detection of 5-HT-SO<sub>4</sub> in human plasma samples was developed. Second, a pilot phase involving four healthy volunteers was executed. Finally, nine healthy volunteers were selected for the main study, where a basal plasma level of 5-HT-SO<sub>4</sub> was measured before and after serotonergic stimulation of the central nervous system. One h after stimulation, six study subjects showed a decrease in 5-HT-SO<sub>4</sub> levels, while three subjects showed an increase. This was the first study in which naturally occurring 5-HT-SO<sub>4</sub> was detected by liquid chromatography-mass spectrometry (LC-MS/MS) in the samples of human plasma obtained from healthy volunteers. The method developed was specific to the measurement of 5-HT-SO<sub>4</sub> and opens up new possibilities to evaluate minor pathways or serotonin metabolism by minimally invasive methods.

**Keywords:** serotonin, serotonin O-sulphate, biomarkers, depression

## 1. Introduction

For several decades, it is noted that biomarkers are playing an increasingly important role in drug discovery and development from target identification and validation to clinical application, thereby making the overall process a more rational approach.

Indisputably, serotonin (5-HT) plays a significant role in the course of depressive disorders, and majority of the drugs developed interferes with this pathway. Therefore, we decided to ascertain the metabolic processes to find perspective areas of research based on experience gathered so far. The most exploited laboratory biomarker methods investigating central nervous system

(CNS) processes use the cerebrospinal fluid (CSF) as far as it is site specific and reveals local processes. Thus, measurement of indoleamine metabolites in the cerebrospinal fluid (CSF) remains an analytical evaluation method of drug efficacy during clinical trials. Nevertheless, due to patient's safety concerns and clinical convenience, a method employing less invasive approach is highly appreciated by health care professionals.

Thus, our objective was based on critical literature review approach and practical liquid chromatography-mass spectrometry (LC-MS/MS) method implementation to investigate a possibility to use 5-HT-SO<sub>4</sub> as a potential CNS-specific serotonin metabolism biomarker based on a less invasive laboratory method suitable for clinical and pharmacological studies.

## 2. Literature evidence related to 5-HT-SO<sub>4</sub> appearance in animal and humans

Sulphonate conjugation was first described in 1876 and has since been shown to be a significant pathway in the biotransformation of many neurotransmitters.

During a critical literature review, our special attention was drawn to the final phase of 5-HT degradation by sulphotransferases (SULT) and the end product of biotransformation, 5-HT-SO<sub>4</sub>. We identified 66 papers and excluded 51 papers that did not contain data related to 5-HT-SO<sub>4</sub>. In total, 15 papers were included in the final review with 10 analysed in terms of outcomes.

We found that during the last century, a sulphation of serotonin was described by Kishimoto and a final product of such biotransformation, serotonin-O-sulphate, was found [1]. Furthermore, during later years in animal experiments, it was approved that 5-HT-SO<sub>4</sub> is the final product of serotonin metabolism which is rapidly excreted from the organism [2, 3]. Later, a similar compound was found in human urine, cerebrospinal liquor and platelets [4–6]. During recent years, some research was done with marine molluscs determining 5-HT-SO<sub>4</sub> in their nervous system [7, 8]. Findings showed that the serotonin metabolite 5-HT-SO<sub>4</sub> forms from 5-HT uptake and metabolism in central ganglia and other structures of nervous system but not in haemolymph itself.

**Table 1** summarises the evidence related to 5-HT-SO<sub>4</sub> appearance in animals and humans.

As shown in **Table 1**, 5-HT-O-SO<sub>4</sub> was intensively investigated by G.M. Tyce during the 1980s and 1990s. Initially, a considerable amount of acid-hydrolysable conjugates of dopamine, nor-epinephrine (NE) and 5-HT were detected in lumbar CSF of normal individuals. The amounts of conjugated amines were small in comparison to the amounts of homovanillic acid and 5-hydroxyindoleacetic acid [9]. In a further study performed with CSF from humans and ventriculocisternal perfusion of African green monkeys found that the sulphates of NE, dopamine and 5-HT are present in the CSF of laboratory animals and humans. All amines and metabolites were quantitated by using high-performance liquid chromatography (HPLC) with electrochemical detection. The amounts of sulphated amines in human CSF always greatly exceed the amounts of the free amines [6]. This gave us a preliminary impression of 5-HT-SO<sub>4</sub> site

| Date | Research name  | Results   | Analytical methods applied   |
|------|--|---|--|
| 2009 | Analysis of intact glucuronides and sulphates of serotonin, dopamine, and their phase I metabolites in rat brain microdialysates by liquid chromatography-tandem mass spectrometry | The LC-MS/MS method was validated by determining the limits of detection and quantitation, linearity and repeatability for the quantitative analysis of 5-HT and DA and their glucuronides, as well as of 5-HIAA, DOPAC and HVA and their sulphateconjugates. | LC-MS/MS   |
| 2008 | 5-HT and 5-HT-SO <sub>4</sub> but not tryptophan or 5-HIAA levels in single feeding neurons track animal hunger state  | Changes in levels of 5-HT-SO <sub>4</sub> in the metacerebral giant neurons of <i>Pleurobranchaea californica</i> related to feeding were observed  | Capillary electrophoresis with laser-induced wavelength-resolved fluorescence detection (CE-LIF) |
| 2007 | Serotonin catabolism in the central and enteric nervous systems of rats upon induction of serotonin syndrome   | Serotonin sulphate showed surprisingly large increases in rat intestinal tissues after induction of serotonin syndrome  | <i>Capillary electrophoresis with laser-induced native fluorescence detection (CE-LINF)</i>      |
| 2004 | Systemic serotonin sulphate in opisthobranch molluscs  | 5-HT-SO <sub>4</sub> forms in neural cells. Not detected in haemolymph  | Capillary electrophoresis (CE) system  |
| 2003 | Serotonin catabolism depends upon location of release: characterisation of sulphated and gamma-glutamylated serotonin metabolites in <i>Aplysia californica</i>                    | The pathway of serotonin inactivation with further formation of 5-HT-SO <sub>4</sub> depends upon the type of neuronal tissue subjected to neurotransmitter incubation  | CE-LIF LC-MS   |
| 1988 | Presence of phenolsulphotransferase activity in microvascular endothelial cells: formation of 5-HT-O-sulphate in intact cells  | Existence of phenolsulphotransferase in the endothelial cells and formation of 5-HT-SO <sub>4</sub> verified  | Not available  |
| 1986 | Amine sulphate formation in the central nervous system   | Origin of the central nervous system of amine sulphates (also 5-HT-SO <sub>4</sub> ) in monkeys and humans observed. 5-HT-SO <sub>4</sub> was detected in CSF of monkeys and humans but not in the plasma   | High performance liquid chromatography (HPLC) with electrochemical detection                     |
| 1985 | Free and conjugated amines in human lumbar cerebrospinal fluid   | 5-HT-SO <sub>4</sub> was detected in the CSF of healthy humans  | HPLC with electrochemical detection  |
| 1983 | Exploration of the role of phenolsulphotransferase in the disposition of serotonin in human platelets: implications for a novel therapeutic strategy against depression            | Existence of phenolsulphotransferase in the platelets and formation of 5-HT-SO <sub>4</sub> verified  | The assay technique-purified alveolysin toxin  |
| 1966 | Isolation of serotonin-O-sulphate from human urine   | 5-HT-SO <sub>4</sub> isolated from human urine  | Ion exchange resins  |

**Table 1.** Summary of the evidence found related to 5-HT-SO<sub>4</sub> appearance in animals and humans.

specificity. At the time of above mentioned studies, 5-HT-O-SO<sub>4</sub> could not be detected in the plasma of untreated monkeys and the concentration of 5-HT-O-SO<sub>4</sub> in brain perfusates versus plasma increased after injection of 5-HT sulphate. The ratio of amine sulphate in the brain versus amine sulphate in plasma was greater for 5-HT-O-SO<sub>4</sub> than for DA-O-sulphate at 60 and 100 min after injection. Finally, it was concluded that although 5-HT-O-SO<sub>4</sub> could not be detected in the plasma of monkeys or humans under normal conditions, the 5-HT-O-SO<sub>4</sub> in ventriculocisternal perfusates undoubtedly originates in the CNS [6].

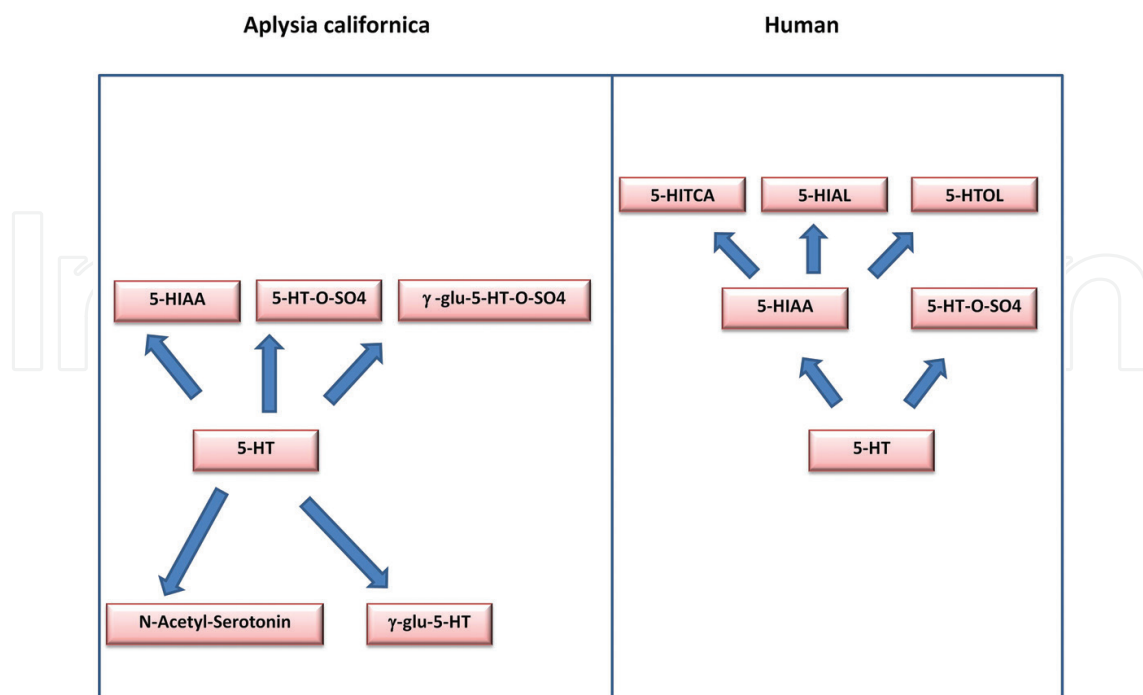
This obstacle inspired us to analyse more recent studies selected during review.

Some research was conducted with marine molluscs determining 5-HT-O-SO<sub>4</sub> in their nervous system [7, 8].

In one such research done in 2003, incubation of neuronal tissue of *Aplysia* revealed three novel 5-HT catabolites. **Figure 1** summarises the metabolism of 5-HT found in *Aplysia* central ganglia compared to human.

As seen from **Figure 1**, there is no difference of 5-HT-O-SO<sub>4</sub> formation between humans and molluscs.

As shown in **Table 2**, the 5-HT-O-SO<sub>4</sub> can be detected in CNS and its formation though is site specific, and later animal studies confirm detection in periphery. Moreover, as far similar sulphotransfases exist in sea molluscs and mammals, an equal process should be theorised for humans [10]. Also, literature evidence exists that 5-HT-SO<sub>4</sub> was proposed to be measured in the animal urine or plasma which makes it to be relative simply detected by HPLC with various detectors [11].



**Figure 1.** Metabolites of 5-HT in the marine mollusc *Aplysia californica* versus humans. The novel metabolites are shown for *Aplysia californica*. Only detailed 5-HIAA metabolism is shown for humans.

| Trial              | Quantity of trial subjects | Species        | 5-HT-O-SO <sub>4</sub> qualified | 5-HT-O-SO <sub>4</sub> quantified | 5-HT-O-SO <sub>4</sub> found in nervous system | 5-HT-O-SO <sub>4</sub> found outside nervous system | Conclusions   |
|--------------------|----------------------------|----------------|----------------------------------|-----------------------------------|--|---|---|
| Tyce et al. [9]    | 22                         | Humans         | No. defined as conjugate of 5-HT | No. defined as conjugate of 5-HT  | No. defined as conjugate of 5-HT               | No  | CSF contains 5-HT conjugate   |
| Tyce et al. [6]    | 12/4                       | Humans/animals | Yes                              | Yes                               | Yes  | No  | 5-HT-O-SO <sub>4</sub> originates from CNS  |
| Stuart et al. [7]  | N/A                        | Animals        | Yes                              | No                                | Yes  | No <sup>c</sup>                                     | 5-HT-O-SO <sub>4</sub> formation is nervous-system specific                                     |
| Stuart et al. [8]  | 11                         | Animals        | Yes                              | Yes                               | Yes  | Yes   | 5-HT-O-SO <sub>4</sub> forms in the nervous system but not in haemolymph itself                 |
| Uutela et al. [12] | N/A                        | Animals        | Yes                              | Yes                               | Yes  | No  | 5-HT-O-SO <sub>4</sub> in rat brain microdialysates was analysed using a direct LC-MS/MS method |

**Table 2.** Summary of trials involving 5-HT-O-SO<sub>4</sub>.

Summarising the literature review, there is evidence of similarities between human and animal metabolic pathways, and as far as there is literature evidence of site-specific 5-HT-SO<sub>4</sub> formation in animals, we can extrapolate the same to humans.

We noted that historically used methods assumed highly invasive approach of CSF sample collection from human subjects. Therefore, search for a minimally invasive method has significant clinical benefit. Moreover, the latest 5-HT-SO<sub>4</sub> research revealed experience with LC-MS application for such a purpose.

### 3. The latest findings related to 5-HT-SO<sub>4</sub> appearance in humans

The evidence in the scientific literature justifies the decision to employ detection of 5-HT-SO<sub>4</sub> in clinical practice. As far as there was no literature data particularly on 5-HT-SO<sub>4</sub> detection by LC-MS/MS in the human plasma, we initiated a development of the chromatography method based on literature evidence related to detection of similar compounds such as indoleamines in the human plasma. The objective concerning analytical procedure was to demonstrate that it is suitable for its intended purpose—qualitative detection of 5-HT-SO<sub>4</sub> in the human plasma.

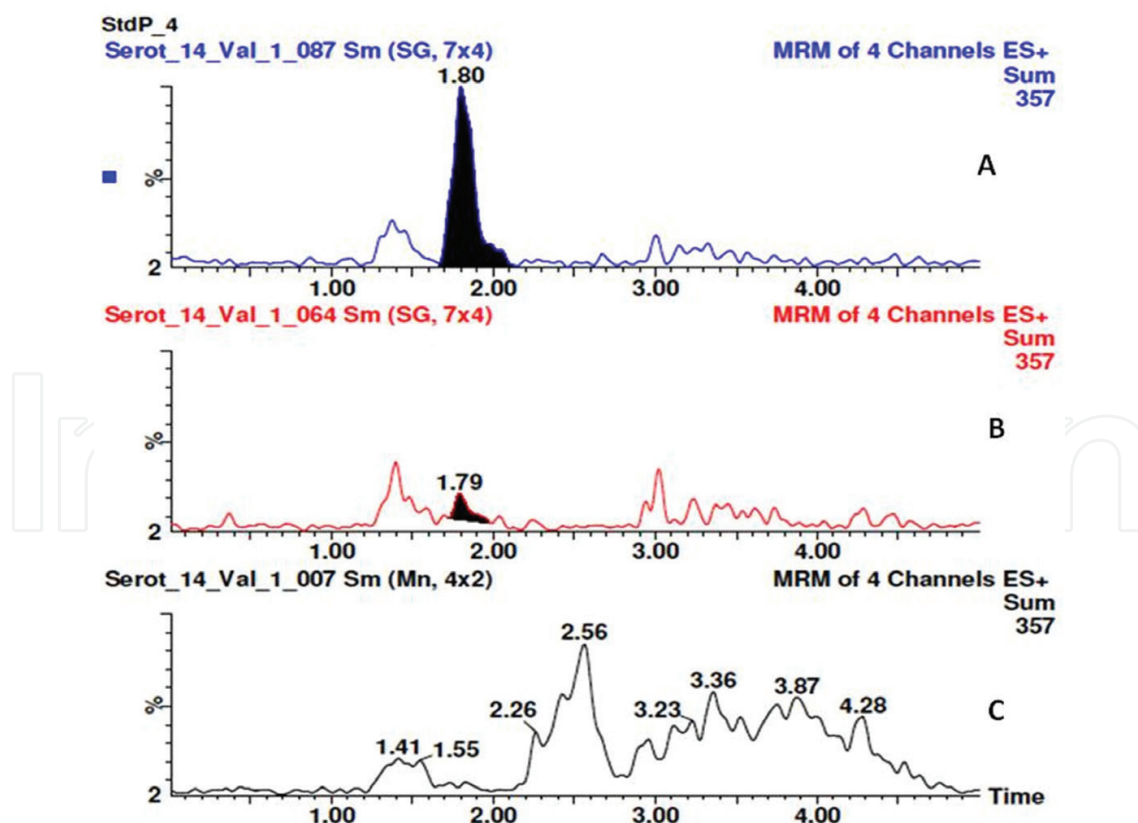
Tandem mass spectrometric analysis (MS/MS) was made in a positive-ion mode (ESI+). The electrospray ionisation of 5-HT-SO<sub>4</sub> was weak. Thus, for the further quantitative analysis, a following ion transition was used: (257>>160) + (240>>160).



Specificity of the method was assessed visually by comparing multiple reaction monitoring (MRM) chromatograms of plasma sample spiked with serotonin O-sulphate, samples of plasma and purified water. As seen in **Figure 2**, in the plasma-based calibration standard (A) and plasma (B), some 1.79–1.80 min retention time peaks can be observed [13]. In the plasma-based calibration standard and “pure” plasma, some peaks with a retention time of 1.79–1.80 min were observed. The purified water samples treated similarly do not show such signals (C). This signal might be induced by native content of serotonin sulphate found in plasma samples. Conclusion was reached because in the analytical solution made of 5% serum albumin, such a signal was not seen [13]. For the MRM chromatograms, the test solution of 5% serum albumin (buffered to pH = 7 in a phosphate buffer) was prepared.

The results obtained lead to conclusion that the method developed is specific to the compound of interest, 5-HT-SO<sub>4</sub>.

The results obtained lead to the conclusion that the method developed is specific to the compound of interest, 5-HT-SO<sub>4</sub>, in samples of human plasma. The linearity of detection was evaluated three times in different days by analysing calibration standard solutions of 5-HT-SO<sub>4</sub> [13].



**Figure 2.** Chromatograms of 5-HT-SO<sub>4</sub> samples. (A) Plasma standard solution (containing 96 ng/mL of 5-HT-SO<sub>4</sub>); (B) “pure” plasma sample and (C) water.

This method resulted in a linear relationship between concentration of the analyte (from 10 to 225 ng/mL) and a mass spectral signal of 5-HT-SO<sub>4</sub> with a calibration curve correlation coefficient of >0.98.

The optimal detection limit of 5-HT-SO<sub>4</sub> in the plasma sample was determined to be 26.5 ng/mL. Four different concentrations of 5-HT-SO<sub>4</sub> were used for a recovery testing and the method gave correct 5-HT-SO<sub>4</sub> detection results, which were justified by the average level of recovery of the analyte at  $116 \pm 8\%$ . The relatively high interval of recovery can be explained due to the matrix effect [13].

The intra-laboratory accuracy of the method over a 3-day period was characterised by a standard deviation of  $\pm 11.95\%$  [13]. Taking into account above mentioned results, the method was concluded to be a suitable technique for measuring 5-HT-SO<sub>4</sub> in human blood samples.

The findings of method development phase led to the decision to perform the first-in-humans study in order to assess the clinical applicability of the LC-MS/MS method developed, and the studies were designed to quantify intra-individual results using a cohort of healthy subjects. The clinical study had a two-stage design: a pilot study and main Study.

The pilot study confirmed that the peaks with retention time 1.79–1.80 min are detected in the samples of plasma of healthy volunteers. These peaks corresponded to the signal of 5-HT-SO<sub>4</sub>. One study subject was exposed to oral intake of L-5-hydroxytryptophan (5-HTP) containing food supplement to observe the influence of serotonergic stimulation to 5-HT-SO<sub>4</sub> level. The pilot study proved that the 5-HT-SO<sub>4</sub> could be qualified in plasma samples obtained from healthy volunteers. The increase in 5-HT-SO<sub>4</sub> level after serotonergic stimulation was observed. Unfortunately, all results were below the detection limit of the method and probably due to several matrix-, method-, or analyte-specific reasons.

Our primary interest was to ascertain quantitative differences of basal 5-HT-SO<sub>4</sub> levels, the intra-individual sensitivity of the quantitation as well as detection limit issues obtained in the pilot study on a larger number of subjects.

Thus, after measurement of the basal 5-HT-SO<sub>4</sub> levels in nine subjects, all of them were exposed to serotonergic stimulation with a food supplement containing 100 mg of 5-HTP and a second blood sample was analysed. In six study subjects, a decrease in 5-HT-SO<sub>4</sub> levels was observed 1 h after 5-HTP ingestion. Three subjects, however, showed an increase in 5-HT-SO<sub>4</sub> 1 h after 5-HTP ingestion. Out of nine study subjects and one pilot subject, an increase in 5-HT-SO<sub>4</sub> 1 h after 5-HTP ingestion was observed in three women and one man, respectively. The others—five men and one woman—showed decrease. A graphical chart of study results is shown in **Figure 3**.

The outcome of our studies is that we developed a liquid chromatography method, which is specific to the measurement of 5-HT-SO<sub>4</sub> in the samples of human plasma. It is the first time when 5-HT-SO<sub>4</sub> was detected in the plasma obtained from healthy volunteers [14]. The sensitivity of the LC-MS/MS method to detect intra-individual changes of the compound in the healthy volunteers undergoing supplementation with 5-HTP was observed, but the majority of results were below detection limit [13].



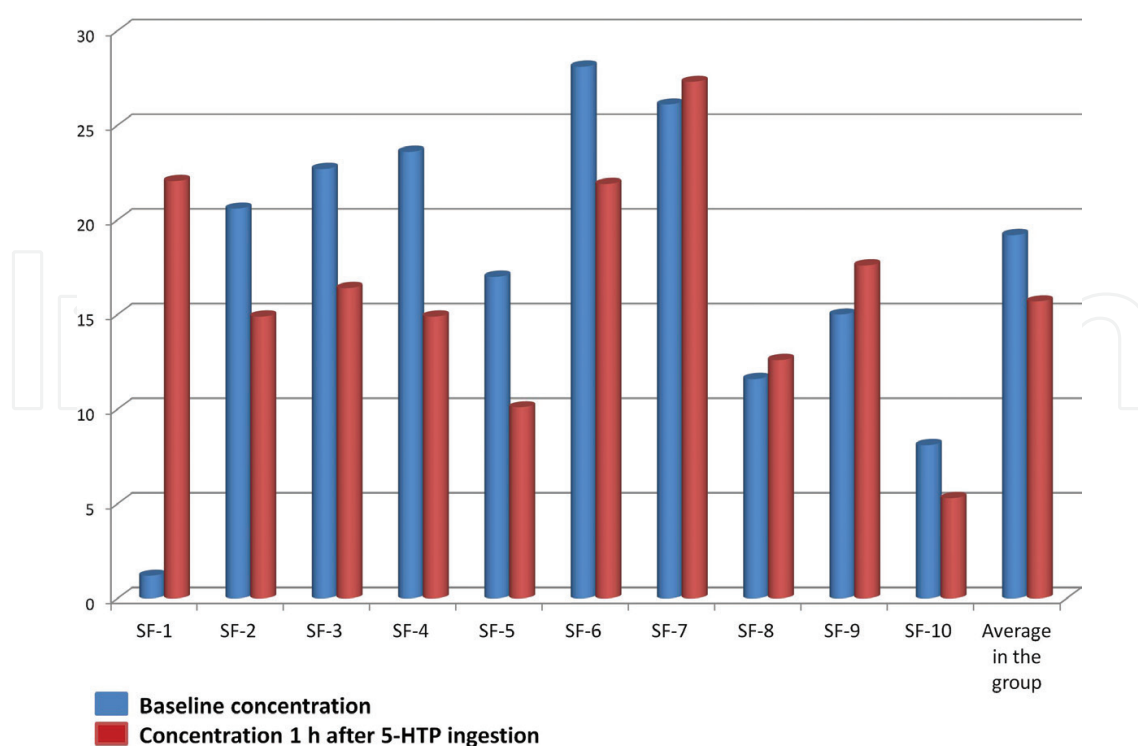


Figure 3. A graphical view of studies results.

## 4. Conclusions and discussion

This chapter ascertains a possibility for  $5\text{-HT-SO}_4$  to be employed as a potential serotonin metabolism biomarker based on a less invasive laboratory method. Based on critical literature review,  $5\text{-HT-SO}_4$  is identified as a potential 5-HT metabolism biomarker to be detected by a minimally invasive approach in human plasma. The novel LC-MS/MS method, which is specific to the measurement of  $5\text{-HT-SO}_4$  in the human plasma, has been developed [13]. It was the first time when  $5\text{-HT-SO}_4$  was detected in the plasma obtained from healthy volunteers [13]. However, the clinical applicability of the method was not justified as the majority of results were below detection limit of 26.5 ng/mL [13].

Concerns regarding the issue of whether  $5\text{-HT-SO}_4$  we found in the plasma has CNS origin or not should be evaluated. It is known that L-amino acid decarboxylase acts both in the periphery and in the CNS which can result with the ingested 5-HTP being converted into serotonin in the periphery of the body too [15], but plasmatic serotonin mostly derived from peripheral tissues is primarily metabolised in the liver to 5-hydroxyindole acetate and then excreted in the urine [16, 17]. Regarding 5-HT locating in the gastrointestinal tract, it is known that once serotonin reuptake transporter (SERT) has brought serotonin into the epithelial cells, it is metabolised to 5-HIAA by monoamine oxidase which is localised to all intestinal epithelial cells [18]. Alternatively, 5-HT released into the lamina propria may enter the portal vein circulation and be detected either as free serotonin or within platelets (via the actions of SERT). As the liver processes the portal circulation, enzymes rapidly degrade the

free 5-HT. The monoamine oxidase degrades about one-third to urine detectable 5-HIAA. The remaining two-thirds of serotonin is degraded to the metabolite 5-HT-O-glucuronide. It should be noted that 5-HT taken up by platelets is protected from degradation in the liver and enters the general blood circulation [18]. Coincidentally, the only sites of 5-HT sulphation identified in humans are the CNS and enteric nervous systems [6, 19] while enteric nervous system 5-HT-SO<sub>4</sub> was found only after induction of serotonin syndrome [19]. Some isoforms of SULT have been shown to have sulphate serotonin [20]. Also, findings in sea molluscs indicate that metabolism of serotonin with further formation of 5-HT-SO<sub>4</sub> depends upon the location of release. Thus, haemolymph 5-HT-SO<sub>4</sub> most probably originates from the nervous system [7, 8]. There is also no doubt about the entrance of ingested 5-HTP into CNS [21]. Also, earlier studies concluded that under normal conditions, the 5-HT-O-SO<sub>4</sub> originates from CNS [6]. The most significant finding was that 5-HT-O-SO<sub>4</sub> freely crosses blood-CSF barrier, so physiological circumstances are not preventing the appearance of CNS-originated 5-HT-O-SO<sub>4</sub> in the venous blood circulation. Therefore, taking into account all aforementioned facts, we are more concerned that 5-HT-SO<sub>4</sub> detected in the study [13] mimics serotonin metabolism in CNS. Future investigations are needed to justify this assumption.

The most disputable outcome of our research is the elevation or reduction of 5-HT-SO<sub>4</sub>. The majority of volunteers from the study phase, six out of nine, had a drop of plasma sulphate concentration [12]. Although there is no data available regarding diurnal rhythmicity of serotonin sulphate levels in the human plasma, we are not able to confirm whether these changes are due to the direct influence of serotonergic stimulation. To investigate the possible link to the health status of study subjects with 5-HT-SO<sub>4</sub> level changes, we performed questioning. Hamilton depression rating scale results revealed mild depression in four subjects. Unfortunately, we cannot make any clinical conclusion related to the correlation between symptoms and laboratory findings due to results below the detection limit, but the trend seems to be very intriguing.

Nevertheless, in the light of literature data, we tend to explain phenomena observed by substrate inhibition of SULT 1A3 [22, 23]. It could be concluded that under normal circumstances, quantity of serotonin synthesised and metabolised is kept under certain limits [20]. Data favouring this is evidence of paradoxical actions of the 5-HTP on the activity of identified serotonergic neurons in a simple motor circuit. It was found that more serotonin did not lead to more potent swim motoraction, implying that serotonin synthesis must be kept within certain limits for the circuit to function properly. Also, alteration of neurotransmitter synthesis can lead to grave consequences for the output of neural networks [24]. Described mechanisms could be taken into account explaining our results. Thus, we hypothesise that a drop of 5-HT-SO<sub>4</sub> in plasma would be related to the overproduction of serotonin, leading to inhibition of SULT 1A3. Elevation of 5-HT-SO<sub>4</sub> was probably a sign of serotonin deficiency, but such an opinion also requires further investigation. The latter would correlate with the experiment made in sea molluscs when hungry animals had significantly higher levels of serotonin and 5-HT-SO<sub>4</sub> than their satiated partners [25]. It remains for future investigations to determine whether serotonin sulphate found in plasma has central nervous system origin and the reason for elevated or lowered 5-HT-SO<sub>4</sub> levels after serotonergic stimulation [13].

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